APPENDIX A

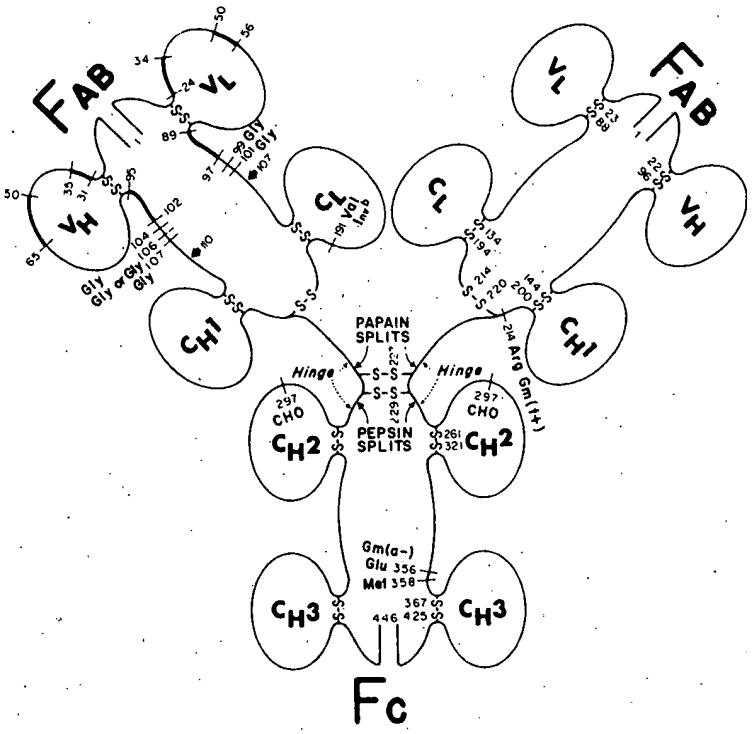


Fig. 11.1. Schematic view of four-chain structure of human IgG_K molecule. Numbers on right side denote actual residues of protein Eu [10, 11]. Numbers of Fab fragments on the left side are aligned for maximum homology; light chains are numbered according to Wu & Kabat [12, 13]. Heavy chains of Eu have residue 52A and 82A, B, C and lack residues termed 100A, B, C, D, E, F, G, H, and 35A, B. Thus residue 100 (end of variable region) is 114 in actual sequence. Hypervariable regions, e.g. complementarity-determining segments or regions, are shown by heavier lines. V_L and V_H denote light and heavy chain variable regions; C_H1, C_H2, and C_H3 are domains of constant region of heavy chain; C_L is constant region of light chain. The hinge region in which two heavy chains are linked by disulphide bonds is indicated approximately. Sites of action of papain and pepsin and locations of a number of genetic factors are given. From 14.

portions of antibody could be found even in the α_2-globulin region. Immunoelectrophoretic analysis [9] showed the γ-globulins to migrate over a very wide range from the α_2 region to the slow γ region. One could recognize more restricted heterogeneity in antibody populations by movement over a narrower range. The heterogeneity of IgM and of the later discovered IgA was also much more apparent from immunoelectrophoretic patterns. Physico-chemical analysis of heterogeneity, paucidispersity or monoclonality of antibodies may now be carried out by more powerful methods such as isoelectric focusing. It should be recognized that the physico-chemical parameters generally do not provide information about the antibody combining site.

The various immunoglobulins may be digested by enzymes to give Fab, $F(ab')_2$ and Fc fragments (Porter, Nisonoff), or they may be reduced with mercaptoethanol giving monomeric IgM. On complete reduction they were shown to have a two-chain structure: a light chain with about 214 and for IgG a heavy chain with about 446 amino acid residues (Fig. 11.1). In IgG the light chains were shown to have two domains and the heavy chains four, each domain being associated with distinct functions and properties with the V_L and V_H domains determining antibody specificity. Thus sequence variations in the other domains may be determining factors of some physico-chemical heterogeneities. Conversely, differences in specificity of antibodies of the same class or subclass are reflected in sequence differences of the V domains, and these can affect the physico-chemical findings. In addition, immunochemical differences recognized as allotypic and isotypic have been correlated with sequence differences and associated with particular domains. The *a* allotypes of the rabbit involve differences in the framework of the V_H domain; other allotypes are associated with one or another C domain [15].

Had homogeneous antibodies been produced to various antigens, even in the 1930s and 1940s, it would have been difficult or impossible, from the immunochemical standpoint, to establish that all molecules of a given antibody preparation contained identical antibody combining sites, although electrophoretic homogeneity was readily seen. It required the development of equilibrium dialysis [16] and the analysis of data by Gaussian, Scatchard, or Sips analysis to provide evidence of valence and of site heterogeneity or homogeneity [17]. This has now been supplemented by fluorescence quenching and enhancement, fluorescence polarization, ultracentrifugal techniques, electron spin resonance, nuclear magnetic resonance, etc. [4].

Recognition of the existence of homogeneous anti-

Chapter 14
Preparation and purification of active fragments from mouse monoclonal antibodies

P. PARHAM

Why fragment antibodies?

Partial proteolysis of immunoglobulins has been useful in both the analysis of immunoglobulin structure and the application of specific antibodies to the study of numerous biological systems, including the immune system itself.

The immunoglobulin molecule as exemplified by IgG is bifunctional. It brings together specific antigens and non-specific mechanisms that inactivate and degrade antigens. The binding sites are located in different domains of the molecule that can be separated by proteolytic cleavage with retention of activity [1]. When using antibodies as tools, one can sometimes take advantage of their bifunctionality. An example is immunoprecipitation, where solid-phase protein A is used to bind soluble antigen–antibody complexes via the Fc portion of the antibody. In other cases one only wants an antigen binding reagent, and binding of antibody through sites other than the antigen combining site can lead to artefacts and incorrect interpretation of results. To give one example: Geier & Cresswell [2] showed that inhibition of the human mixed lymphocyte reaction by rabbit antisera to class II MHC antigens was Fc dependent as F(ab')$_2$ fragments caused no inhibition. These results suggested that inhibition was due to elimination of the cells by phagocytic or cytotoxic cells rather than to blocking of stimulation by class II molecules as had been suggested by their initial experiments [3].

A second feature of immunoglobulins that can be either useful or a problem is multivalency. *In vivo* it allows Ig to aggregate antigen, rendering it more susceptible to phagocytosis. Multivalency also increases the strength of antigen–antibody interactions over that provided by the single combining site affinities [4]. In immune precipitation or radioimmune assays, these effects are often beneficial and are crucial in the reactions of polyclonal antisera. It may in fact be desirable to use mixtures of monoclonal antibodies against epitopes of an antigen in order to increase the functional affinity [5,6]. In studies of the functions of cell-surface molecules, multivalent interaction may perturb the system in an undesirable fashion. Capping of cell surface molecules often occurs and in some cases results in endocytosis and loss of the molecule of interest from the cell surface. Receptors can be stimulated by cross-linking and an anti-receptor antibody may mimic the ligand inducing a change in the cell's metabolism and state of differentiation. For example, this is observed with antibodies against the antigen receptors of B and T lymphocytes [7,8].

Cells are often used in competitive radioimmune assays designed to assess the topology and relationship of epitopes recognized by a panel of monoclonal antibodies directed against a cell surface molecule. When multivalent cold competitors are used, one can observe competition between antibodies that are directed against quite distinct sites [9,10]. Such problems can be significantly reduced and sometimes eliminated by using monovalent Fab fragments.

Quantitative estimations of antibody affinity and antigenic site number are also more easily and accurately carried out with Fab fragments providing they are of sufficiently high affinity [11, and Chapter 38 by Mason & Williams in this volume]. This is often not the case when monoclonal antibodies have been identified with screening assays using cells, viruses,

and other multivalent antigens. In some cases the avidity is high but the affinity is low and a practical level of binding cannot be obtained with Fab. For this type of antibody, quantification can be more accurately carried out with $F(ab')_2$ because one can in that case often assume that binding is exclusively bivalent [10]. For some antibodies, Fab and $F(ab')_2$ fragments bind with higher affinity to cells than IgG [12].

The original experiments that showed the cleavage of rabbit and human Ig into Fab, $F(ab)_2$, Fc, and other fragments are described by Stanworth & Turner [13] in the previous edition of this book and will not be discussed further. Methods for fragmentation of mouse Ig have not been so well described. This can be attributed to three reasons. First, mice have not been generally used for the preparation of antisera because of their small blood volume. They were mainly the preserve of mouse immunogeneticists needing alloantisera. Second, the fragmentation conditions that worked for rabbit and human were not always directly applicable to mouse Ig. For polyclonal antisera this affect is compounded by the large difference in protease susceptibility between the subclasses of mouse IgG. Third, the only mouse immunoglobulins available in large amounts were myeloma proteins of unidentified or uninteresting specificity. These were of most use for primary structural analysis where fragmentation after chain separation and denaturation was used. Despite these problems a number of useful procedures were established and these will be cited where appropriate. The impetus to develop general methods for making mouse immunoglobulin fragments has come with the availability of mouse monoclonal antibodies of predefined specificity [14] which are now replacing polyclonal rabbit and goat antisera as the research reagent of choice. Unfortunately the number of publications on fragmentation is still small. Few of the methods are tried and proven in the hands of many and procedures for obtaining all of the potentially useful fragments have not been described. However, the state of the art is definitely better than as previously described in collections of immunological methods.

Equipment

The procedures described here can all be done with the basic equipment of a biochemistry laboratory:
ultraviolet spectrophotometer;
fraction collector;
37 °C water bath;
chromatography columns;
stirred cells for concentration by ultrafiltration using PM 10 membranes (Amicon);
vertical slab gel electrophoresis apparatus for SDS-PAGE;
power pack.
An isocratic HPLC system and TSK 3000SW column (Beckman Instruments) are useful for monitoring proteolytic digests and the purity of preparations by size exclusion chromatography, but they are not essential. The sensitivity of the system will be mainly determined by the UV detector and the wavelength used. We use a Beckman variable UV wavelength detector (model 165) set at 219 nm. This detects at the level of 1 μg.

Most chromatographic steps involve DEAE-cellulose (DE-52, Whatman) and various Sephadex gel filtration (size exclusion) resins (Pharmacia). The technical aspects of the chromatography will not be described in detail as both manufacturers provide comprehensive instructions in their catalogues and ancillary literature (*Improved Techniques with Advanced Ion Exchange Celluloses* and *Advanced Ion Exchange Celluloses Product Guide*, by Whatman; *Gel Filtration Theory and Practice*, by Pharmacia). A useful practical and theoretical guide to basic biochemical procedures is provided by Cooper T.G. *The Tools of Biochemistry* [15]. Other more specialized sources of help are: *Protein Purification Principles and Practice*, Scope R. (1982), Springer-Verlag, New York. Gel filtration chromatography, Fischer L. (1980), in Vol. 1, part II of *Laboratory Techniques in Biochemistry and Molecular Biology*, (eds. Work T.S. & Burdon R.H.), Elsevier/North–Holland. Electrophoresis of proteins in polyacrylamide and starch gels, Gordon A.H. (1978), in Vol. 1, part I of *Laboratory Techniques in Biochemistry and Molecular Biology*, (eds. Work T.S. & Burdon R.H.), Elsevier/North–Holland. Regnier [16] provides an introduction and references to the application of HPLC to the analysis and purification of proteins.

Analysis of fragmentation

Much of the earlier difficulty encountered in this area was due to the indirect methods of analysing the fragmentation of IgG, i.e. immunodiffusion and immunoelectrophoresis. This is no longer a problem as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion high-pressure liquid chromatography (HPLC) allow one to rapidly analyse the cleavage of the heavy chains, the cross-linking of polypeptides by disulphide bridges, and the size of the fragments produced.

SDS-PAGE

This method of analysis separates polypeptide chains under denaturing conditions according to size. Practi-

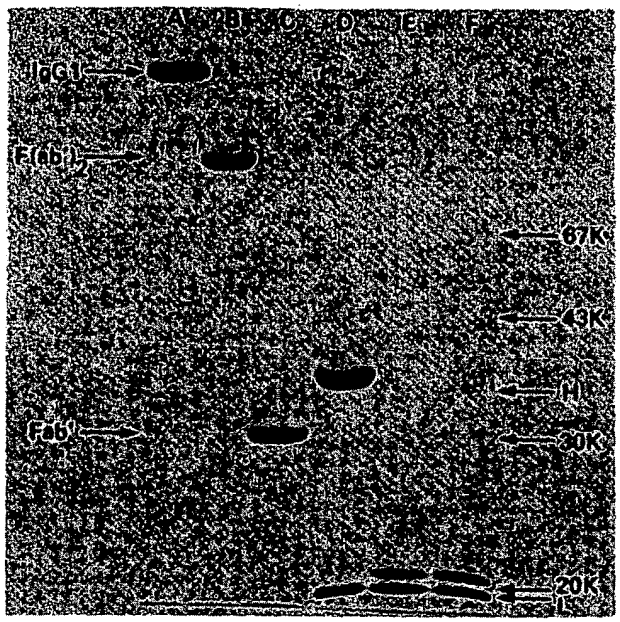


Fig. 14.1. SDS-PAGE under non-reducing (A,B,C) and reducing (D,E,F) conditions of purified IgG (A,D), $F(ab')_2$ (B,F), and Fab' (C,F) preparation. All are derived from the MB40.3 monoclonal IgG1 [27].

cal details and references can be found in ref. 15. The distance migrated is proportional to the logarithm of the relative molecular mass. The samples for electrophoresis can be prepared in the presence or absence of the reducing agent β-mercaptoethanol (β-MeSH) which permits analysis of the disulphide bonds between polypeptides. For immunoglobulins this comparison is useful, as shown in Fig. 14.1. Without β-MeSH, IgG migrates as a single protein band with apparent relative molecular mass (Mr) of $\simeq$ 150 000; with β-MeSH, IgG migrates as two protein bands corresponding to the heavy chain (Mr $\simeq$ 50 000) and the light chain (Mr $\simeq$ 25 000). $F(ab')_2$ under non-reducing conditions migrates as a single band of Mr $\simeq$ 110 000 and when reduced is a doublet of bands at Mr $\simeq$ 25 000. The lower band of the doublet corresponds to the light chain and the other is the NH_2-terminal half of the cleaved heavy chain (Fd). Fab or Fab' migrates under non-reducing conditions as a single band of Mr $\simeq$ 50 000, and when reduced gives a pattern identical to $F(ab')_2$. Fc under non-reducing and reducing conditions gives a single band of Mr $\simeq$ 25 000 migrating slower than the Fab doublet. Thus it is possible to unequivocally identify and assay for IgG, $F(ab')_2$, Fab and Fc.*

* For simplicity we shall use $F(ab')_2$ for all bivalent antigen binding fragments that lack Fc, irrespective of the class of immunoglobulin or the enzyme used for digestion. Fab' will refer to univalent fragments made from $F(ab')_2$ by reduction. Fab will refer to univalent antigen binding fragments arising directly from digestion of IgG.

Size exclusion HPLC

The critical step in the preparation of antibody fragments is the generation of $F(ab')_2$ from IgG (see Fig. 14.5). This results in a change of relative molecular mass from $\simeq$ 150 000 to $\simeq$ 110 000. Size exclusion columns using soft gels such as Sephadex poorly resolve IgG and $F(ab')_2$ and have been of limited use in analysing and separating enzymatic digests containing these fragments. The time for separation of a single sample (12–36 h) has also limited their use for analytical purposes. High-pressure size exclusion columns combine an improved separation of IgG from $F(ab')_2$ with a significant decrease in the time required for an analysis (20 min–2 h) (Fig. 14.2). The small bore of the column provides the system with higher sensitivity and amounts of 1–5 μg can be detected. This system will distinguish IgG, $F(ab')_2$, and Fab and can be used for monitoring the course of enzymatic digests (Fig. 14.3).

The analytical experiment

The only generalization that one can state with some certainty is that all monoclonal antibodies are unique. It is essential, therefore, to perform analytical experiments on any untested monoclonal antibody in order to assess whether a given procedure will work. This will also allow minor adjustments to be made in order to maximize yields, ensure complete degradation of IgG, etc. Analytical experiments are carried out under

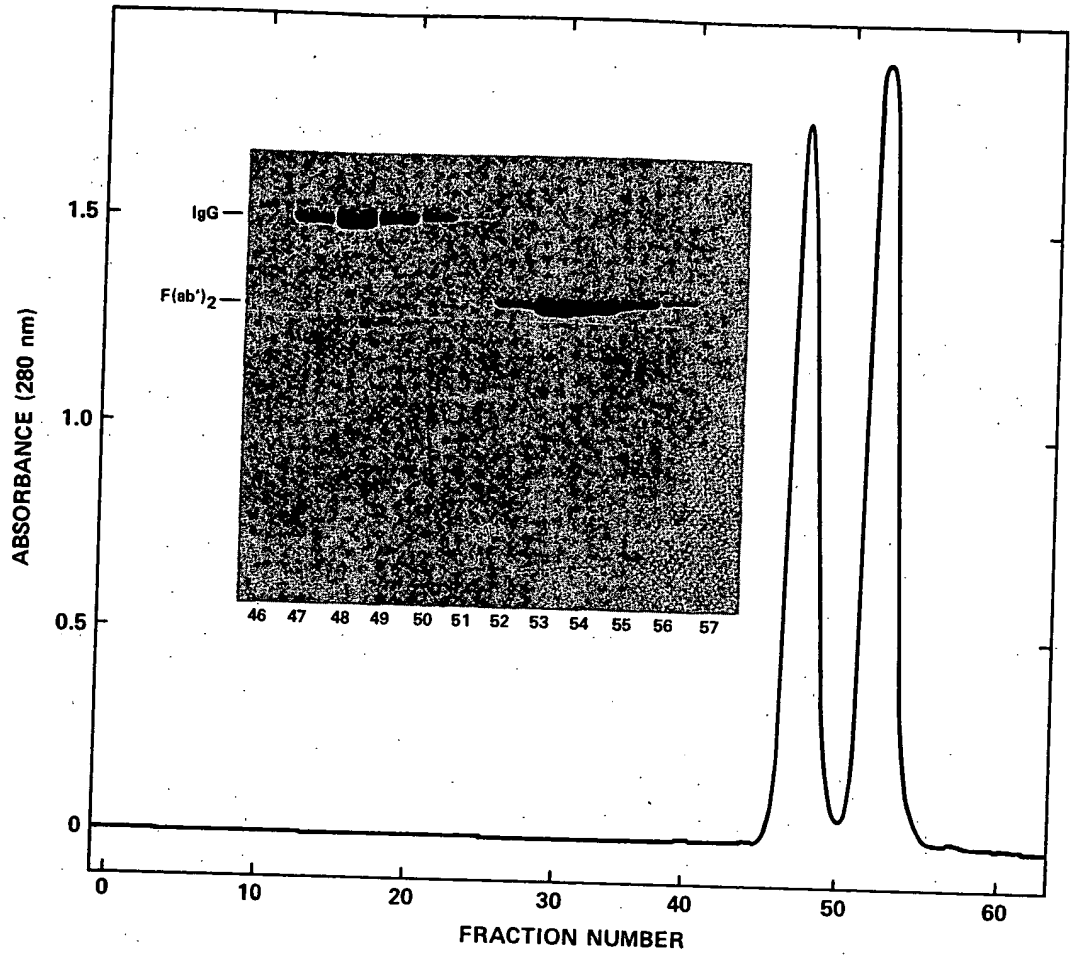


Fig. 14.2. Separation of F(ab′)$_2$ and IgG by size exclusion HPLC. Two milligrams in 250 μl of a 50% mixture of monoclonal IgG1 and F(ab)$_2$ were chromatographed on a 0.75 × 60 cm Spherogel TSK 3000 SW column with a 0.75 × 10 cm Spherogel TSW SW precolumn. A Beckman 324MP liquid chromatograph and 165 UV-variable wavelength detector were used. The column buffer, 0.1 M-phosphate, pH 7.0, was prepared from glass distilled filtered water and refiltered after solute addition through a 0.45 μm Millipore filter. The buffer was degassed by passage of a stream of helium. The sample was centrifuged for 10 mm at 170 000 *g* in a Beckman airfuge and filtered through a 0.2 μm membrane filter of regenerated cellulose using an MF-1 microfiltration system (Bioanalytical Systems). Flow rate was 0.2 ml/min at a pressure of $\simeq$ 138 kPa and 0.32 ml fractions were collected. Ten microlitres of fractions 46–57 were analysed by SDS-PAGE on a 7% gel under non-reducing conditions [29].

conditions that are identical to those anticipated for a preparative experiment except that small amounts of material are used and one parameter, usually time, is varied. Example: in the author's laboratory, F(ab′)$_2$ is routinely prepared by pepsin digestion of IgG1 using the following conditions:

[IgG] = 1–2 mg/ml
[Pepsin] = 25 μg/ml
Temperature = 37 °C
Buffer = 0.1 M-citrate, pH 3.5.

For an uncharacterized antibody, a digestion with 400 μg IgG1 is set up and at times 0, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, and 48 h, 20 μg aliquots are removed and the reaction terminated by raising the pH to > 7.0. At the end of the time course all the samples are analysed by SDS-PAGE under reducing and non-reducing conditions. Untreated IgG and an authentic F(ab′)$_2$ from another monoclonal IgG1 are analysed on the same gels for comparison. This analysis will show if the new antibody is undergo-

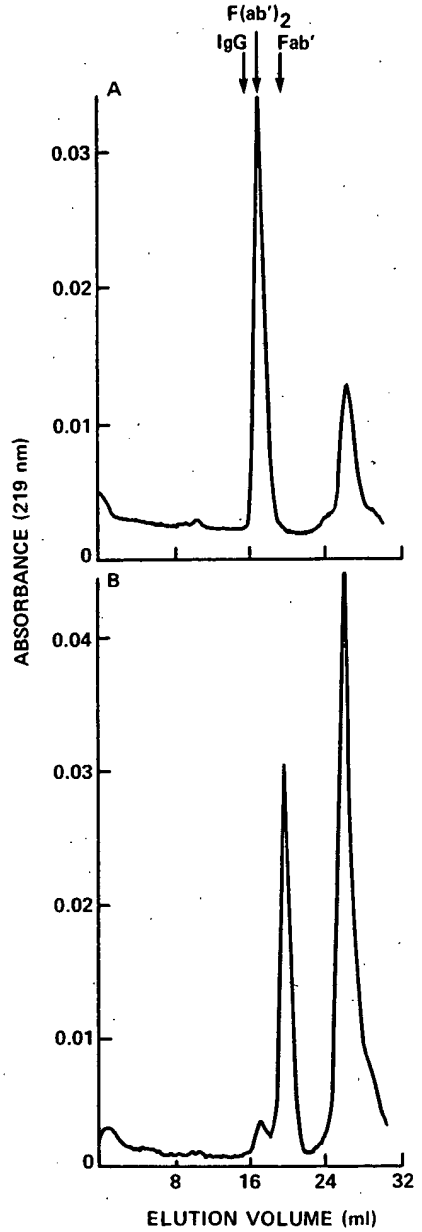


Fig. 14.3. Analysis by size exclusion HPLC of the products of pepsin digestion of IgG1. Digestion was for 8 h, at 37 °C in 0.1 M-citrate, pH 3.5, with IgG at 1 mg/ml and pepsin at 25 μg/ml. In panel A, a 2 μl sample was analysed by HPLC as described in the legend to Fig. 14.2 except that a flow rate of 1.2 ml/min was used and protein was detected at 219 nm. A 25 μl aliquot of the digest was made, 10 mM in cysteine and incubated for 1 h at 37 °C before analysis of 2 μl as shown in B. The elution positions of authentic samples of IgG, $F(ab')_2$, and Fab' from the same monoclonal antibody are shown. The low Mr peak eluting at 26.4 ml comprises small peptides, amino acids, sodium azide, and in B, cysteine [27].

ing 'normal' cleavage and allow an appropriate time of digestion to be selected. If cleavage is not 'normal', then a more detailed investigation of the characteristics of the antibody is called for. This would involve similar analytical experiments in which other parameters, e.g. pH, enzyme, temperature, are varied.

When conditions for a preparative experiment have been chosen, the course of the digest is monitored, either while it is happening with HPLC or after the fact by SDS-PAGE. At the end of the digestion one can remove an aliquot for analysis and rapidly freeze the remainder without inactivating the enzyme. If analysis shows the reaction is not complete, the sample can be thawed and the digestion continued. When the reaction is complete, the sample is thawed and the protease inactivated. One major cause of differential susceptibility to cleavage is antibody isotype and this will be discussed in some detail. Little consideration will be given to differences resulting from allotype or the strain of mouse from which a monoclonal antibody is derived. This is because of insufficient information. Most studies have been with Ig from BALB/c mice and there is limited information from a few other strains. One must, however, anticipate significant heterogeneity due to the strain of origin, because the hinge region is both the site of proteolysis and of considerable allotypic variation (Table 14.1) [17]. An additional source of variability is the state of the Ig from which the fragments are prepared. Antibodies purified by affinity methods such as protein A or anti-immunoglobulin columns, that involve elution with acid or base, are more likely to have undergone partial denaturation and be more susceptible to proteolysis; it is similar for samples that have been lyophilized, heat inactivated or repeatedly freeze-thawed.

Basic strategy

All classes of immunoglobulin are Y-shaped molecules made up of two heavy and two light chains. Secreted forms of IgM and IgA are pentameric and dimeric oligomers of these structures. The fragments of monoclonal antibodies that one commonly wants are $F(ab')_2$ and Fab; the bivalent and monovalent antigen binding fragments. A strategy for obtaining them that would also yield Fc is shown in Fig. 14.4. This involves proteolytic cleavage to give $F(ab')_2$ and Fc, then mild reduction and alkylation of $F(ab')_2$ to yield Fab'. Ideally this chapter should provide methods for doing this for each class of immunoglobulin. In reality it can be done for IgG1, the most abundant class, and somewhat less effectively for IgG2a and IgG3. For Ig2b it has not been possible to make $F(ab')_2$ and the same is possibly true for IgD and IgA. In these cases Fab is the primary cleavage product. Limited studies

Table 14.1. Amino acid sequences of hinge regions of mouse IgG

	216				220										230									
IgG2a^b	Glu	Pro	Arg	Val	Pro	Ile		Thr	Gln	Asn	Pro	Cys	Pro	Pro	His	Gln	Arg	Val	Pro	Pro	Cys	Ala	Ala	Pro
IgG2a^a	Glu	Pro	Arg	Gly	Pro			Thr	Ile	Lys	Pro	Cys	Pro	Pro	Cys					Lys	Cys	Pro	Ala	Pro
IgG2b^a	Glu	Pro	Ser	Gly	Pro	Ile	Ser	Thr	Ile	Asn	Pro	Cys	Pro	Pro	Cys	Lys	Glu	Cys	His	Lys	Cys	Pro	Ala	Pro
IgG1^a	Val	Pro	Arg	Asp								Cys	Gly		Cys	Lys	Pro	Cys		Ile	Cys	Thr		
																						△		

	240									250
IgG2a^b	Asp	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe
IgG2a^a	Asn	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe
IgG2b^a	Asn	Leu	Glu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe
IgG1^a		Val	Pro	Glu	Val	Ser	Ser	Val	Phe	Ile	Phe
							▲				

The sequences are derived from analysis of IgG_{2a} [60] and IgG_1 [61] proteins and of DNA derived from the IgG_1 [62], IgG_{2a} [17,63], and IgG_{2b} [64] genes. Blanks are the results of alignment of the sequences to maximize homology. Cysteine residues involved in the inter-heavy chain disulphide bonds are underlined; the residues at position 227 bond to the light chain. The site of pepsin cleavage of IgG_1 at pH 4.0 as determined by Svasti & Milstein [61] is indicated by ▲. △ denotes the boundary between the hinge region and the C_H2 domain as defined by the start of the C_H2 coding sequence. The numbering is that of Schreier *et al.* [17]. IgG2a^a, IgG2b^a, and IgG1^a are from BALB/c mice and IgG2a^b is from C57BL/6 mice.

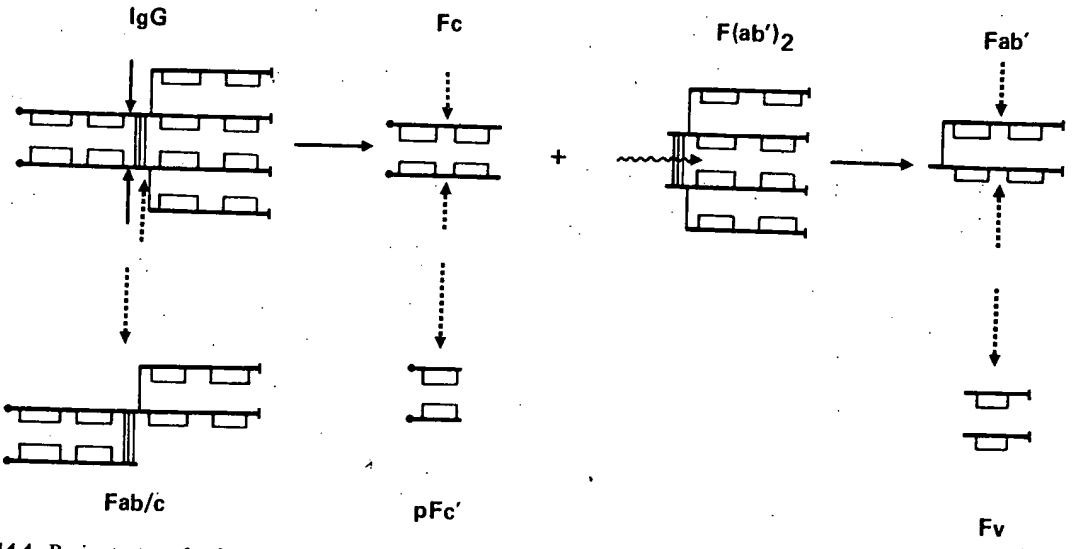


Fig. 14.4. Basic strategy for fragmenting IgG. Sites of cleavage are indicated by solid arrow for reactions from left to right and dashed arrows for reactions from top to bottom.

on IgE and IgM suggest $F(ab')_2$ can be made from these isotypes. Apart from the three common Ig fragments—$F(ab')_2$, Fab, and Fc—other fragments have been characterized in studies of particular classes of immunoglobulin, i.e. pFc' from IgG1, Fv from IgA, and Fab/c from IgG2b. These are also indicated in Fig. 14.4 and will be discussed.

Preparation of $F(ab')_2$ from IgG

The first step in our basic strategy is the preparation of $F(ab')_2$.

'There is no satisfactory procedure of preparing mouse $F(ab')_2$ because mouse IgG tends to precipitate from solution at the acidic pH required for pepsin action' [18].

'Peptic digestion of mouse IgG has not provided large fragments in acceptable yields, possibly because mouse IgG unfolds at the low pH required for activity of the enzyme' [19].

'There are earlier reports in the literature on making mouse $F(ab')_2$ with pepsin but we have been repeatedly unable to reproduce these results' [20].

These three quotations were indicative of the circulating immunological wisdom on this subject at the time (~ 1979) that mouse monoclonal antibodies were entering common usage. The absence of any discussion on this topic by other collections of immunological methods, including earlier editions of this volume, reinforced these views. Pepsin had been used to produce $F(ab')_2$ from IgG fractions of mouse antisera [21,22,23,24] using conditions that Nisonoff *et al.* [25] had optimized for rabbit IgG. Incomplete digestion was a major problem and extensive purification was required to obtain $F(ab')_2$ free of contaminating IgG. In 1969, however, Gorini [26] had shown that mouse IgG subclasses are differentially susceptible to proteolysis by trypsin, papain, and pepsin. Their order of sensitivity—IgG2b > IgG2a > IgG1—has been confirmed [27,28,29] and IgG3 is between IgG2b and IgG2a in pepsin sensitivity. With hindsight it seems likely that the pepsin digestions of heterogeneous mouse IgG fractions resulted in destruction of IgG2b and IgG3, incomplete cleavage of IgG1 to $F(ab')_2$, and generation of $F(ab')_2$ and Fab from IgG2a. In addition to the problem of purifying $F(ab')_2$ from the mixture of products was that of selective loss of activity specifically associated with an isotype. With monoclonal antibodies, this is not a problem as the stringency of digestion can be adjusted according to isotype.

The structures of the hinge region for the classes of mouse IgG are quite different, as shown in Fig. 14.5 and Table 14.1. The most striking difference is the arrangement of the disulphide bridge between the heavy and light chains of IgG1 compared to IgG2a, IgG2b, and IgG3. As this affects the hinge region where proteolytic attack to generate Fab and $F(ab')_2$ takes place, it is not surprising that these differences result in significant differences in susceptibility to proteolysis and the nature of the cleavage.

IgG1

IgG1 is most resistant to proteolysis but can be cleaved in good yield to either $F(ab')_2$ + Fc (thiol-free pre-activated papain)*, Fab + Fc (papain + cysteine) or $F(ab')_2$ (pepsin). These are similar to the classic fragmentation patterns of rabbit IgG [13]. The fragments are relatively stable to subsequent degradation.

* The bivalent antigen binding fragment produced by papain has been called $F(ab)_2$, e.g. see ref. 10.

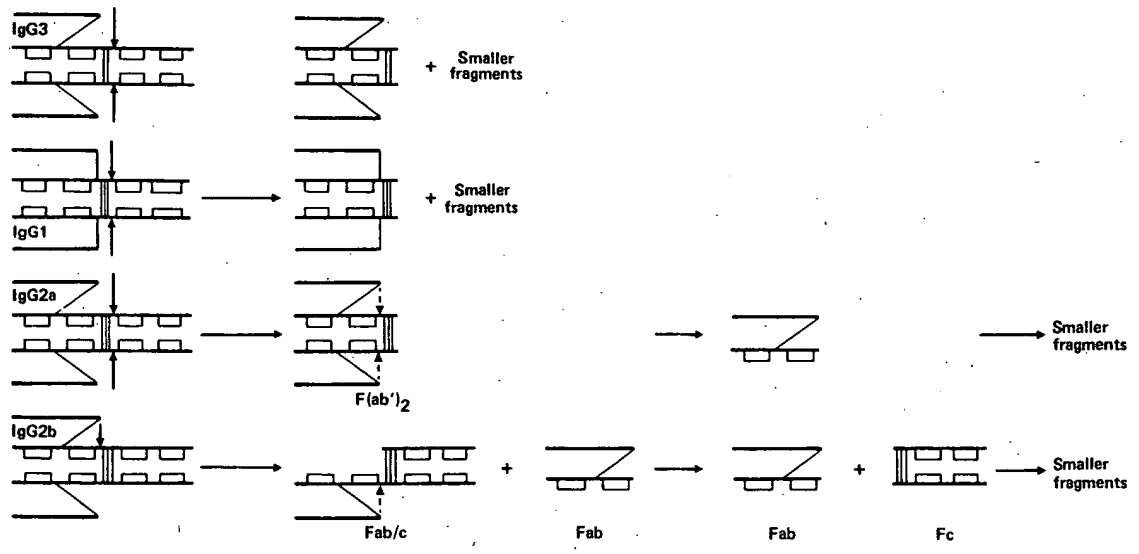


Fig. 14.5. Arrangement of the disulphides in the four subclasses of mouse IgG and the patterns of fragmentation obtained with pepsin [27,28].

Preparation of F(ab')$_2$ and Fc from mouse IgG1 with papain

Gorini *et al.* [26] showed that papain in the presence of 10 mM-cysteine rapidly and completely digested myeloma IgG1 into Fab and Fc. When cysteine was omitted, digestion was slower and a fragment of Mr ~115000 was obtained. It is also obtained when monoclonal IgG1 is digested with thiol-free pre-activated papain [29] and is a bivalent binding fragment similar to the F(ab')$_2$ produced with pepsin.

Papain digestion has the following characteristics: (1) it yields an intact Fc in addition to F(ab')$_2$ or Fab; (2) it can be performed at a higher pH than that used for pepsin; (3) relatively large amounts of enzyme are used.

Materials

Papain (Worthington), 10 units/mg protein, supplied as ammonium sulphate slurry, 20–50 mg/ml.
Sephadex G-25 superfine (Pharmacia).
DEAE cellulose (DE-52 Whatman).
L-Cysteine free base (Sigma).
Iodoacetamide (Sigma).

Procedure

1 IgG1 (10–100 mg) at 1–10 mg/ml in a buffer containing 0.003 M-EDTA, 0.1 M-acetate, pH 5.5. If Ig is stored in a relatively weak buffer, e.g. PBS or 0.025 M-Tris-HCl, pH 7.5, then add 1 M-acetate buffer, pH 5.5, to a final concentration of 0.1 M and adjust the pH by addition of 1 M-acetic acid with rapid mixing. No thiols or reagents that react with thiols, e.g. iodoacetamide, iodoacetic acid, *N*-ethyl maleimide, must be present.

2 Pre-activation of papain. A Sephadex G-25 superfine column is poured in a 10 ml disposable plastic pipette at room temperature and equilibrated with 0.003 M-EDTA, 0.1 M-acetate, pH 5.5. Twice the amount of papain required for digestion is added as slurry to 0.5 ml of 0.003 M-EDTA, 50 mM-cysteine, 0.1 M-acetate, pH 5.5, and incubated for 30 min at 37 °C. The mixture is applied to the G-25 column and 1 ml fractions are collected. When ten fractions have been collected, their absorbance at 280 nm is measured. Papain elutes in the void volume of the column and has an extinction coefficient of $E^{1\%}_{280} = 2.5$ for a standard 1 cm cuvette. The peak fraction is taken for digestion of Ig.

3 Pre-activated, thiol-free papain is added to the IgG solution at a ratio of 1:20 by weight and incubated in a screw-top tube at 37 °C in a water bath, for a period that has been determined in an analytical trial. At this point, a sample of the digest is taken for analysis by SDS-PAGE or HPLC and the remainder is rapidly frozen in an ethanol/dry-ice bath and stored at −70 °C. If significant amounts of IgG remain, the digest is thawed and incubation at 37 °C continued. An additional aliquot of pre-activated papain can also be added if the rate of digestion is slow. If the reaction is complete, the digest is thawed and the enzyme inactivated by addition of 0.1 M-iodoacetamide to a final concentration of 0.025 M. The pH is adjusted to 7.5 by addition of 3.5 M-Tris-HCl, pH 8.6. The digest is allowed to stand for 30 min at room temperature to ensure complete inactivation. It is possible to obtain at this point a sample that contains F(ab')$_2$, Fc, inactivated papain, small peptides, and no residual IgG. For some experiments no further purification is required apart from dialysis into a suitable buffer. The following procedures are to separate and purify F(ab')$_2$ and Fc. They will also eliminate residual IgG.

4 The digest is dialysed extensively against 0.005 M-Tris-HCl, pH 7.5, at 4 °C. A typical sample will be of 10 ml and this will be dialysed for 32 h against one litre, with three changes of buffer.

5 During dialysis a 5–10 ml column of DEAE-cellulose is made in a 10 or 15 ml plastic syringe and washed with two litres of 0.005 M-Tris HCl, pH 7.5. All subsequent procedures are at 4 °C.

6 The dialysed sample is applied to the DEAE column and washed through with the same buffer. Fractions of 5 ml are collected and the absorbance at 280 nm monitored. For many IgG1, the F(ab')$_2$ will flow through the column (Fig. 14.6). When the absorbance of the fractions has reached a stable value that is close to background, the column is eluted with a 400 ml linear gradient from 0 to 0.1 M-NaCl in the same buffer. A flow rate of 20–30 ml/h is used and 2 ml fractions collected. Finally the column is purged with 0.5 M-NaCl in the same buffer. All fractions are analysed spectrophotometrically and selected ones by SDS-PAGE. The order of elution from the column is F(ab')$_2$, IgG, Fc and a good separation of these three components is usually possible. An example showing the most common pattern that the author has observed is in Fig. 14.6. For some antibodies the F(ab')$_2$ binds to the DEAE column and is eluted as a peak in the first part of the gradient. This will generally give a purer preparation of F(ab')$_2$ than for antibodies where the F(ab')$_2$ flows through the DEAE column along with papain and other contaminants.

7 If SDS-PAGE or HPLC analysis indicate that further purification of F(ab')$_2$ or Fc fraction is required, a size exclusion column is used (Fig. 14.7). The choice of column depends upon the amount of material and the nature of the contaminants. For F(ab')$_2$ that has flowed through the DEAE column,

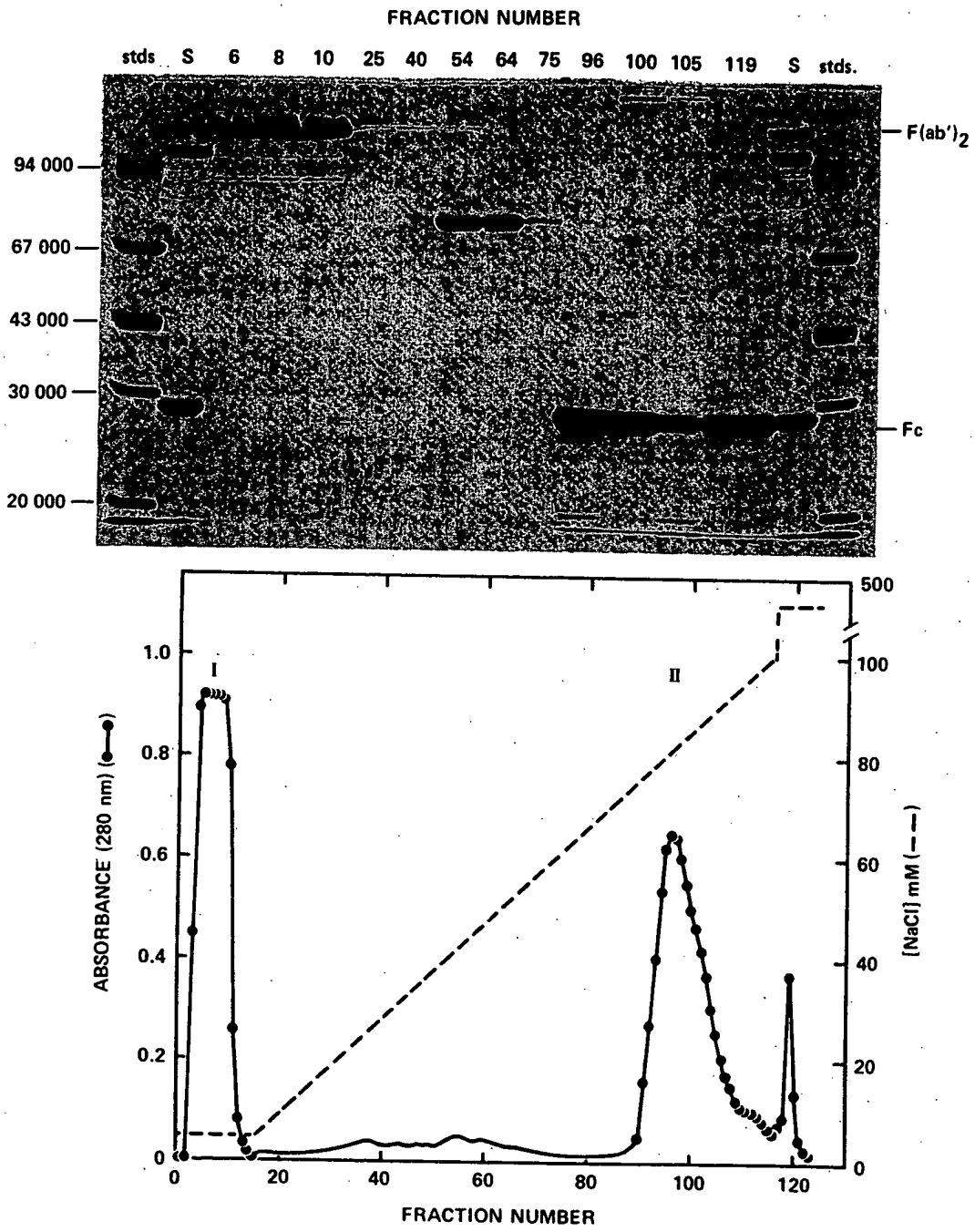


Fig. 14.6. Separation of $F(ab')_2$ and Fc from IgG1 by DEAE cellulose chromatography. A sample containing approximately equal amounts of $F(ab')_2$ and Fc (60 mg total in 60 ml) were dialysed against 0.005 M-Tris-HCl, pH 7.5, and applied to a 5 ml column of DE-52 equilibrated in the same buffer. The sample was washed through with buffer and 5 ml fractions (#1–15) collected. The column was eluted with a 400 ml linear gradient from 0 to 0.1 M-NaCl and then purged with 0.5 M-NaCl. Two ml fractions were collected (#16–123) and assayed spectrophotometrically at 280 nm (lower panel) and by SDS-PAGE under non-reducing conditions (upper panel). S is the sample applied to the column and stds. are relative molecular mass standards. $F(ab')_2$ was recovered in peak I and Fc in peak II. Residual IgG would elute in the middle of the gradient. The band of mobility between $F(ab')_2$ and the 94 000 relative molecular mass standard is a proteolytic artefact of gel sample preparation due to residual active papain in the sample [29].

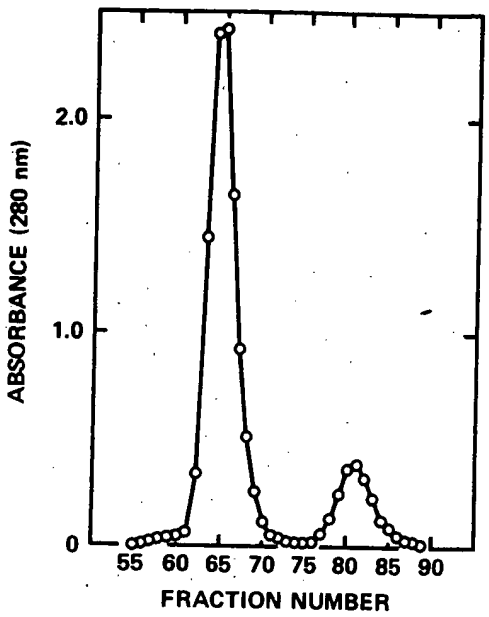
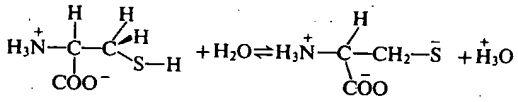


Fig. 14.7. Separation of $F(ab')_2$ from Fab by size exclusion chromatography. An IgG1 was digested with thiol-free pre-activated papain and purified by DEAE cellulose chromatography. The flow-through fractions contained $F(ab')_2$ and some Fab. These fractions (90 mg protein) were concentrated to 15 ml and applied to columns consisting of Sephadex G-75 superfine (5 × 50 cm) followed by Sephadex G-100 superfine (5 × 50 cm) equilibrated in PBS containing 0.02% NaN_3. The column was pumped at a flow rate of 0.5 ml/min, and 10 ml fractions were collected and assayed for absorbance at 280 nm. Analysis by SDS-PAGE showed the major peak, fractions 62–69, was $F(ab')_2$ and the minor peak, fractions 78–83, was Fab'. A single column of Sephadex G-100 superfine (5 × 100 cm) gives a similar separation [29].

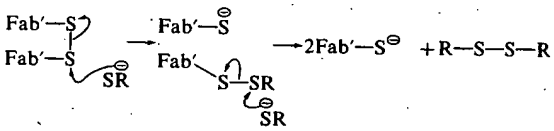
papain and peptides are the usual contaminants. A column of Sephadex G-100 (2.5 × 100 cm) equilibrated in PBS is used and the sample is first concentrated to 5–10 ml by ultrafiltration in an Amicon stirred cell with a PM10 membrane. Fc is often contaminated with $F(ab')_2$ and IgG1 and it can also be cleaned up on the Sephadex G-100 column. When $F(ab')_2$ is contaminated with IgG1, use a Sephadex G-200 column of similar size. Protein A-Sepharose columns cannot be used to efficiently deplete preparations of IgG1 or Fc derived from IgG1.

Reduction and alkylation of $F(ab')_2$ to Fab'

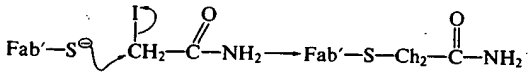
This procedure is used for $F(ab')_2$ from all subclasses. Mild conditions of reduction with cysteine are used to break the H—H inter-heavy disulphide bonds and leave intact the H—L disulphide between the heavy and light chain. Total selectivity is impossible and the conditions can be adjusted depending on whether one prefers to remove residual $F(ab')_2$ from a Fab' preparation with completely intact disulphides or push the formation of Fab' to completion and have partial loss of the H—L disulphide. There is no problem with loss of intra-chain disulphides unless denaturants, e.g. urea and SDS, are present. Fab' fragments with reduced H—L disulphides generally retain their structure and antigen binding activity but are more likely to degrade with time or storage. The rate of reduction is dependent upon the concentration of the anionic form of the cysteine sulphydryl and this increases with pH. Reoxidation to disulphides decreases with pH and the reduced product can be stabilized in the absence of alkylating agents by lowering the pH to below 4. Effective parameters to vary in order to change the degree of reduction are pH, protein concentration and cysteine concentration.



Ionization of cysteine



Reduction of $F(ab')_2$ with cysteine (RS^-)



Alkylation with iodoacetamide

Materials

$F(ab')_2$ is usually in PBS, pH 7.3, or 0.005 M-Tris-HCl, pH 7.5, buffer at this stage and this is appropriate for reduction and alkylation. If it is at a different pH, then dialyse against 0.1 M-Tris-HCl, pH 7.5, or adjust pH with 0.1 M-NaOH or 0.1 M-HCl to pH 7.5.
Fresh 0.1 M solution of cysteine base (Sigma) in 0.1 M-Tris-HCl, pH 7.5.
Fresh 0.1 M-solution of iodoacetamide (Calbiochem) in 0.1 M-Tris-HCl, pH 7.5.

Procedure

1 Add cysteine to $F(ab')_2$ to a final concentration of 0.01 M, mix well, and incubate at 37 °C for 2 h.

2 Add iodoacetamide to reduced $F(ab')_2$, to a final concentration of 0.15 M. Incubate at room temperature for 30 min.

3 Apply to a column of Sephadex G-100 (2.5 × 100 cm) equilibrated in PBS to separate Fab′ from $F(ab')_2$ and reagents.

Notes

The iodoacetamide solution must be well buffered, otherwise it can lower the pH and cause precipitation of Fab′.

Alkylation can be similarly performed with iodoacetic acid if one wants to introduce a negative charge into the Fab′.

With small quantities of material where extensive purification is not possible, reduce at pH 8.0 and remove reagents with a small 10 ml column of Sephadex G-25 equilibrated in PBS.

The extent of reduction can be determined by HPLC before alkylation (Fig. 14.3).

Chemical Modification of Proteins (Means G.E. & Feeney R.E. (1971), Holden-Day, Inc., San Francisco) discusses theory and practice of reduction, alkylation, and other modification procedures.

Pepsin digestion of IgG1 to give $F(ab')_2$

Pepsin, trypsin, and elastase can all be used in addition to papain, to produce $F(ab')_2$ from IgG1 [26,27,28,30]. The conditions of pH required for each enzyme vary and in cases where a monoclonal antibody is unstable at a pH, an alternative condition for digestion can be found. The action of pepsin on IgG1 is particularly sensitive to pH in the range pH 4–4.8 and digestion with a high yield of $F(ab')_2$ can be obtained at pH 4.1 or below (Table 14.2).

Table 14.2. Effects of pH on cleavage of IgG1 by pepsin

pH	% of IgG1 cleaved to $F(ab')_2$
4.0	59
3.9	71
3.8	73
3.7	79
3.6	84
3.5	100

MB40.3 IgG1 at 1.7 mg/ml in 0.1 M-citrate buffers was degraded with pepsin (25 μg/ml) for 8 h at 37 °C. The percentage of cleavage was calculated from the relative areas of the IgG and $F(ab')_2$ peaks [27].

Materials

Pepsin (Worthington).

Procedure

1 IgG1 at 1–10 mg/ml is made 0.1 M in citrate buffer by addition of 1.0 M-citrate, pH 3.5. The pH is adjusted to 3.5 with 0.1 M-HCl or 0.1 M-NaOH. Lyophilized pepsin as supplied is freshly dissolved at 1 mg/ml in 0.1 M-citrate, pH 3.5, and added to IgG1 to give a final concentration of 25 μg/ml.

2 Incubate at 37 °C until there is no residual IgG1. Times of up to 48 h have been used with no major loss of the $F(ab')_2$ product [27]. The reaction is terminated by raising the pH to > 7.0 with 3.0 M-Tris HCl, pH 8.6. Any precipitate is removed by centrifugation at 10 000 *g* for 30 min.

Notes

For some purposes, further purification may not be necessary as $F(ab')_2$ is the only large molecular weight product. Dialysis against PBS removes small peptides and reagents and provides a physiological buffer. Reduction and alkylation to give Fab′ can be done at this stage.

DEAE cellulose chromatography as described for the papain product and/or size exclusion chromatography on Sephadex G-200, G-150 or G-100 are used depending on the nature and extent of the contaminants and the chromatographic properties of the IgG1 on DEAE-cellulose as determined during its purification.

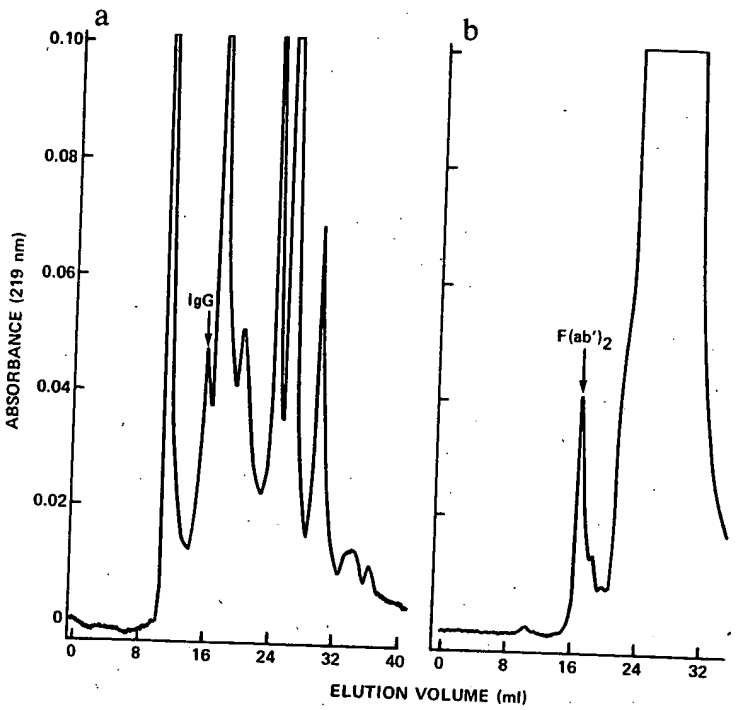
The reaction can be carried out within the pH range 3.5–4.1.

A precipitate is often formed during digestion and most of it redissolves on raising the pH at the end of the reaction.

Due to the stability of $F(ab')_2$ from IgG1, it has been possible to exhaustively digest hybridoma culture supernatants and ascites fluids with pepsin in this way and obtain preparations of $F(ab')_2$ (Fig. 14.8). With purification on DEAE cellulose and Sephadex columns the $F(ab')_2$ preparations from ascitic fluid are comparable in purity to those obtained from purified IgG. A variable contamination of material with Mr ≃65 000 is found in preparations of $F(ab')_2$ from hybridoma supernatants even after DEAE-cellulose and Sephadex G-100 chromatography [27].

IgG2b gives Fab/c, not $F(ab')_2$

IgG2b is most sensitive to proteases. Conditions that are suitable for controlled cleavage of IgG1 or IgG2a



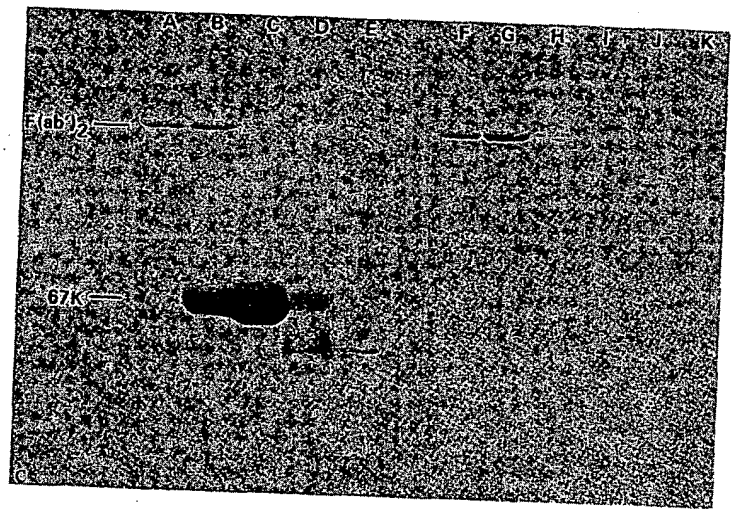


Fig. 14.8. Preparation of $F(ab')_2$ from pepsin digests of IgG1 containing ascites fluids and supernatants. Size exclusion HPLC of: (a) 2 μl of ascites fluid; and (b) 2 μl of ascites fluid after 48 h digestion with pepsin under the conditions described in the legend to Fig. 14.3. $F(ab')_2$ can be similarly detected in digests of cell culture supernatants. Panel C shows the results of purifying $F(ab')_2$ from pepsin digests of IgG1 containing cell culture supernatants. The digests were fractionated by DEAE cellulose and then by Sephadex G-100 (1 × 55 cm column) chromatography. One millilitre fractions were collected from the G-100 column and consecutive fractions through the excluded peak of material analysed by SDS-PAGE under non-reducing conditions. A–E and F–K are preparations from supernatants containing two different monoclonal IgG1 to illustrate the variable presence of contaminants of Mr ≃ 65 000–70 000 [27].

can lead to complete destruction of IgG2b [26,27,28]. The primary cleavage is on the NH_2-terminal side of the inter-heavy chain disulphide bonds to give Fab and this can occur 'spontaneously' with IgG2b preparations that are stored at 4 °C. $F(ab')_2$ fragments have, therefore, not been obtained with any protease. One potential solution to this problem is to isolate 'switch-variants' of IgG2-secreting hybridomas that secrete another class of heavy chain [31] (see Chapter 108). In this way one can retain the combining site specificity while altering the pattern of proteolytic cleavage. Fab and Fc can be obtained in good yield with papain (see page 14.16) or pepsin at pH 4.3–5.0.

We examined the proteolysis of IgG2b with pepsin, papain, trypsin, chymotrypsin, and elastase and the fragmentations were similar. During the early period of a digestion Fab and a fragment of Mr ≃110 000 (non-reducing conditions) are produced concomitantly. It is easy to confuse this fragment with $F(ab')_2$ unless analysis on reducing SDS-PAGE is done. This

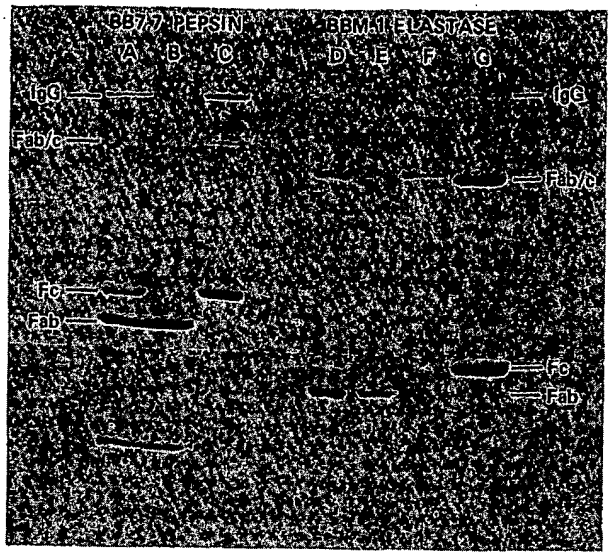


Fig. 14.9. Fractionation of fragments derived from IgG2b on a protein A-Sepharose column. BB7.7-IgG2b (5 mg in 7.5 ml of 0.1 M-acetate, pH 4.8) was digested for 8 h at 37 °C with 5 μg/ml pepsin. BBM.1-IgG2b (22 mg in 22 ml of 0.1 M-Tris-HCl, pH 8.8) was digested for 8 h at 37 °C with 10 μg/ml elastase. The pH of the digests was adjusted to pH 8.0 and they were then applied to columns (2 ml) of protein A-Sepharose 4B CL. The columns were washed extensively with PBS and then eluted with 0.1 M-citrate, pH 3.5. The samples analysed by SDS-PAGE are: for BB7.7, unfractionated digest (A), flow-through (B), and pH 3.5 eluate (C); and for BBM.1, unfractionated digest (D), flow-through (E), PBS wash (F), pH 3.5 eluate (G) [27].

fragment is Fab/c and consists of one Fab and Fc linked by an intact heavy chain (Fig. 14.9). On reducing SDS-PAGE it gives one heavy chain, one light chain and one COOH-terminal heavy chain fragment. During digestion the amount of the Fab/c produced reaches a steady state level as it is further split to yield Fab and Fc [27]. IgG2b characteristically has two heavy chain bands of about equal abundance with different sugar content [32]. The heavy chain of Fab/c is a single band. These results suggest that as found for rabbit IgG [33,34] there is asymmetrical glycosylation of the heavy chains in the hinge region of IgG2b that leads to relative protection of one heavy chain to proteolytic attack. Fab/c is a fragment that eliminates the bivalency of IgG whilst retaining the effector functions of the Fc. It has potential uses in the study of cell surface antigens where modulation by bivalent fragments is a problem and where specific cytolysis mediated by complement or phagocytic cells is required [35]. Fab/c has not been obtainable in high yield as it is an intermediate in the degradation. The best results have been 10–20% of maximum and that was using elastase digestion at pH 8.8 [27]. When pure Fab/c is required it can be advantageous to continue digestion until there is no residual IgG2b. A column of protein A Sepharose is then used to positively select for Fab/c and Fc and this also concentrates the material (Fig. 14.9). Chromatography on Sephadex G-100 is then used to separate Fab/c and Fc. A partial separation of IgG and Fab/c can be obtained with Sephadex G-150 or a TSK 300SW HPLC column (Fig. 14.10).

Elastase digestion of IgG2b

Materials

IgG2b at 1 mg/ml in 0.1 M-Tris-HCl, pH 8.8.
Elastase (Worthington) freshly dissolved at 1 mg/ml in 0.1 M-Tris-HCl, pH 8.8.

Procedure

1 10 μl of elastase per ml of IgG is added. Incubate at 37 °C in a capped tube for 8 h.
2 Add 10 μl of 5% PMSF in absolute ethanol per ml of digest to terminate reaction. Adjust pH to 8.0 by addition of 1 M-Tris-HCl, pH 7.0. Centrifuge at 10 000 *g* at 4 °C for 30 min.
3 Apply supernatant to protein A-Sepharose column (2 ml for 20 mg digest) equilibrated in 0.1 M-Tris-HCl, pH 7.5. Wash with 10 column volumes of 0.1 M-Tris-HCl, pH 7.5. Flow-through contains Fab.
4 Elute with 5 column volumes 0.1 M-citrate, pH 3.5. Collect 1 ml fractions in tubes containing sufficient 1 M-Tris-HCl base to neutralize. Assay eluted fractions by SDS-PAGE and for absorbance at 280 nm. Peak fractions (2 ml) are applied to a column (1 × 66 cm) of Sephadex G-100 superfine equilibrated in PBS. Two peaks of protein are eluted. The first contains Fab/c and the second Fc.

Note

Pepsin can also be used for preparation of Fab/c at a pH of 4.5–5.0 (Fig. 14.11b) [27].

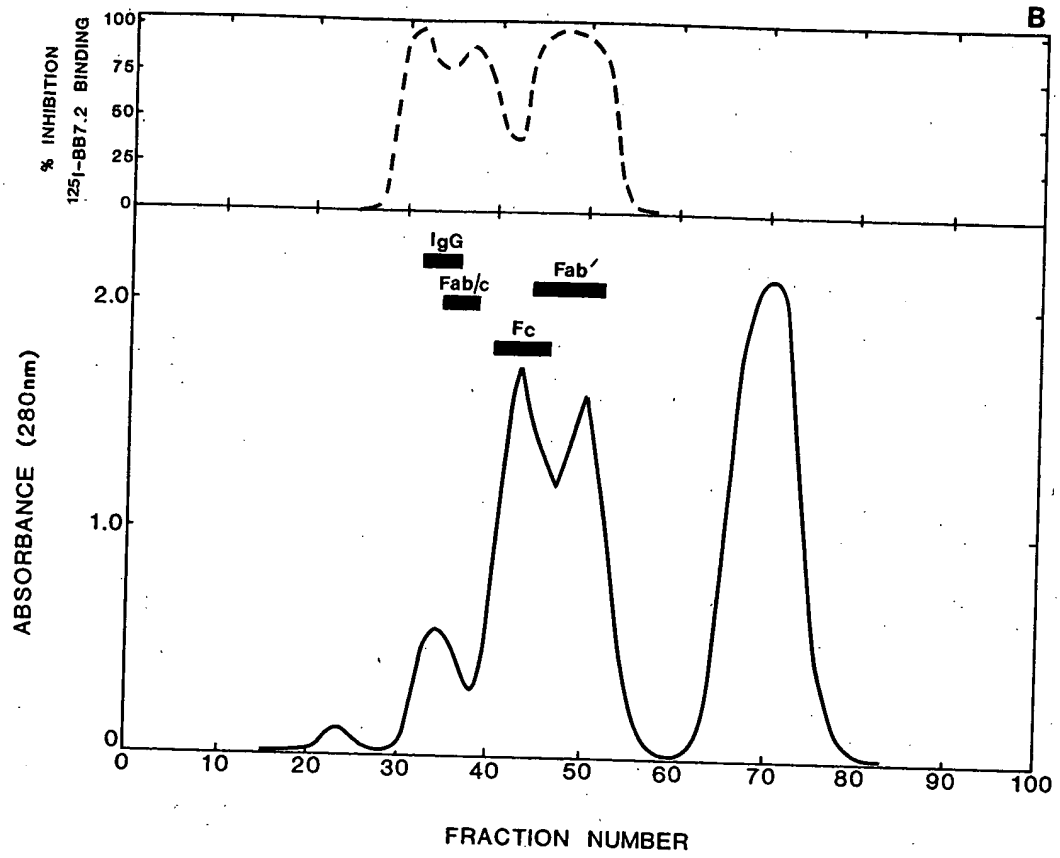


Fig. 14.10. Fractionation of peptic digest of IgG2b on a Sephadex G-150 column. Twenty milligrams of BB7.2 IgG was incubated with 1 mg of pepsin for 8 h at 37 °C in 0.1 M-acetate buffer, pH 4.8. The digest was fractionated on a column (2.5 × 100 cm) of Sephadex G-150 equilibrated in PBS. Ten millilitre fractions were collected and analysed for absorbance at 280 nm (profile in lower panel) by SDS-PAGE (presence of different fragments is indicated in lower panel) and for antigen binding activity (profile in upper panel) [57].

IgG2a

IgG2a is intermediate between IgG1 and IgG2b in sensitivity to proteolytic digestion. Pepsin optimally cleaves IgG2a to $F(ab')_2$ within the pH range 4.0–4.5 (Fig. 14.11a) [27,28]. Under these conditions, Fc is degraded to small peptides. The $F(ab')_2$ product is not resistant to further degradation and with time a fragment having the properties of Fab′ is seen. This is shown in Fig. 14.10 where a time course of pepsin digestion at pH 4.1 is shown. At 8 h $F(ab')_2$ was the major product and about 60% of the IgG was degraded. By 50 h, complete degradation of IgG was accomplished and at this time Fab and $F(ab')_2$ were present in approximately equal molar amounts. Digests of IgG2a can be fractionated by first removing residual IgG on a column of protein A-Sepharose (see page 14.17) and then separating $F(ab')_2$ from Fab′ on a size exclusion column, e.g. Sephadex G-200. On SDS-PAGE with reduction $F(ab')_2$ migrates similarly to the Fab, giving a doublet at ≃25 K. This shows that conversion of $F(ab')_2$ to Fab by pepsin does not involve removal of a large peptide. The most likely explanation is that the secondary pepsin cleavage is on the NH_2-terminal side of the inter-heavy chain disulphide bonds (Fig. 14.5) [27]. We have observed variability in the fragmentation of different IgG2a and have found it advantageous to vary the pH (within the range 4.0–4.5) as well as time in analytical experiments. Digestions are in 0.1 M-acetate buffers with Ig concentrations of 1–2 mg/ml and pepsin at 25 μg/ml. Procedures are otherwise as given for IgG1.

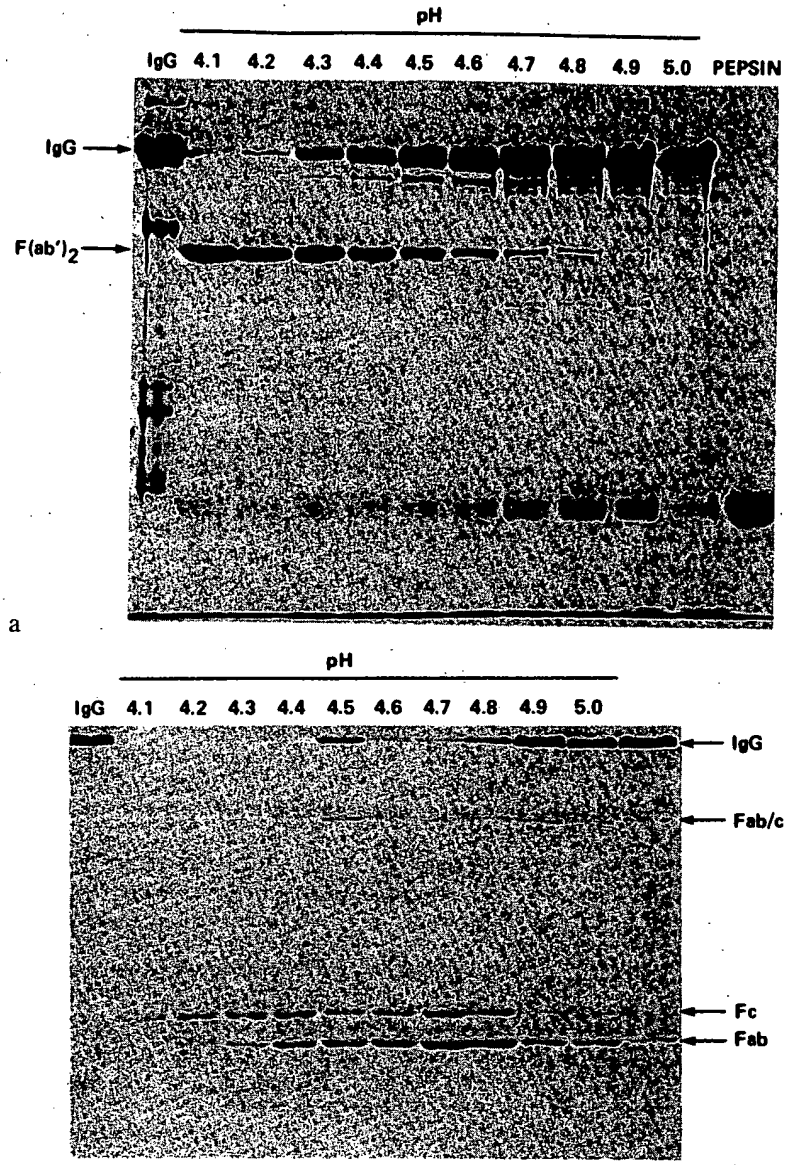


Fig. 14.11. pH dependence of peptic digestion of IgG2a and IgG2b. (a) CVC.7 IgG2a at 0.6 mg/ml in 0.1 M-citrate buffer was incubated with pepsin at 75 μg/ml for 1 h at 37 °C. The pepsin was inactivated by the addition of 1.0 M-Tris-base (74 μl/ml of digest), and samples were analysed on 7.5% SDS-PAGE without reduction. IgG2a and pepsin alone are included as control. (b) BBM.1-IgG2b was incubated with pepsin at pH 4.1–5.1. Incubation was for 8 h at 37 °C in 0.1 M-citrate buffers with IgG at 0.8 mg/ml and pepsin at 2.5 μg/ml. Samples were analysed on 7.5% SDS-PAGE without reduction. IgG alone is included as control [27].

IgG3

Lamoyi & Nisonoff [28] obtained $F(ab')_2$ from two monoclonal IgG3 with the following procedure.

1 Dialyse IgG3 overnight against 0.1 M-sodium acetate, pH 4.0.
2 Lower pH to 4.5 with 2 M-acetic acid.
3 Add pepsin (2× crystallized; Calbiochem) to give enzyme:substrate weight ratio of 1:33.
4 Incubate for 15–30 min at 37 °C.
5 Raise pH to 8.0 with 0.5 M-NaOH.

Table 14.3. Peptic digestion of an IgG3 monoclonal antibody

Period of digestion (min)	% of original antibody activity remaining[a]	% of original activity associated with IgG[b]
15	59	<1
30	45	0
45	34	0
60	29	0

[a] Determined by solid-phase RIA using ^{125}I-labelled, affinity purified rabbit anti-mouse Fab as developing reagent.
[b] Determined by solid-phase RIA using ^{125}I-labelled, affinity purified goat anti-mouse Fc as developing reagent. Reproduced from [28].

As shown in Table 14.3, IgG3 was almost totally gone by 15 min and SDS-PAGE showed that the major product was $F(ab')_2$. The product is quite susceptible to further degradation, putting IgG3 between IgG2a and IgG2b in pepsin sensitivity. It is clearly important to optimize conditions to obtain a good yield of $F(ab')_2$ from IgG3. Lamoyi & Nisonoff [28] favour shorter digestion times and state that 'In our experience any remaining undegraded IgG can be readily removed from $F(ab')_2$ fragments by gel filtration on Sephadex G-150.'

Direct preparation of Fab and Fc from IgG by papain digestion

Fab and Fc can be isolated as major products from papain digests of all classes of mouse IgG when a thiol is included. The resistance to proteolysis is IgG1 > Ig2a > IgG3 > IgG2b and the conditions have to be modified accordingly. We have varied the time of digestion and kept other parameters constant. Mage [36] described a general procedure for IgG of all animal species and the procedures here are mostly derived from that.

Materials

Purified or partially purified preparation of IgG at 1–10 mg/ml. An ammonium sulphate fraction of ascitic fluid is adequate.
10× stock concentration of buffer. Papain is active over a range of pH and buffers of pH from 5–8 have been used in making IgG fragments. Two commonly used buffers are at 1× concentrations: 0.1 M-sodium acetate, pH 5.5; 0.15 M-NaCl 0.01 M-phosphate, pH 7.3. If a monoclonal antibody is unstable or inclined to precipitate under either condition of pH, then it should be possible to find a pH at which the IgG is stable and the papain active.
0.02 M-EDTA (Mallinckrodt) in selected buffer (1×).
0.1 M-cysteine free base (Sigma) in selected buffer (1×)—make solution fresh.
Papain (Worthington), 1–10 mg/ml, freshly diluted in selected buffer (1×).
0.15 M-iodoacetamide (Sigma) in selected buffer (1×).
The digestion is carried out in a screw-cap tube with a volume that is about twice that of the IgG.

Procedure

1 Consider an experiment with 20 ml of IgG at 5 mg/ml. To the IgG solution add 2 ml (1/10 of original volume of IgG) of 10× stock buffer. In general, the IgG solution will not be at extreme pH or have a high buffering capacity and will not significantly change the pH of the added buffer. If this is not the case, the IgG should be first dialysed against the digestion buffer. Alternatively, the pH may be adjusted by addition with rapid mixing of 0.1 M-NaOH or 0.1 M-HCl. It is always worth checking the pH at this stage.
2 Add 1 ml of cysteine solution (1/20 of original volume of IgG).
3 Add 1 ml of EDTA solution (1/20 of original volume of IgG).
4 Add 1 mg of papain to give a weight ratio of 100:1.
5 Cap the tube and mix well by end-over-end rotation. Vortexing or vigorous shaking is not recommended as it causes foaming and denaturation.
6 Incubate in a 37 °C water bath for the time decided on the basis of the analytical experiment. This will generally be between 1 and 8 h. Mix the digest from time to time during the incubation by inverting the tube a few times.
7 Remove from the water bath and add 1 ml of iodoacetamide solution to terminate the reaction. Mix well and leave at room temperature for 30 min to ensure complete alkylation.
Before commencing purification of the fragments, the products of digestion are analysed by SDS-PAGE under reducing and non-reducing conditions. The digest contains unreacted iodoacetamide that will trap β-MeSH in the reducing sample buffer. Therefore additional β-MeSH, to a final concentration of 0.02 M, is added to those samples. A range of amounts is analysed so that minor species are seen in overloaded lanes and major species are resolved in lightly loaded lanes. Precipitation commonly occurs during digestion but rarely involves a major part of the material. Before SDS-PAGE, the aliquots of the digest are spun, and the supernatant and any precipitate analysed

separately. If a major part of a desired product has precipitated, it may sometimes be redissolved by raising the pH to 11.0 by addition of 1 M-NaOH and after a minute bringing the pH back to 7.0–8.0 by addition of a 3 M-Tris-HCl buffer.

Further purification

With IgG1, the reaction can be taken to completion with little loss of product. Residual IgG need not be a contaminant and the major separation required is Fab from Fc, papain, peptides, and reagents. For other classes of IgG, the optimal conditions will give a mixture of products including residual IgG.

DEAE cellulose chromatography

The digest is dialysed against three changes of 0.02% NaN_3 0.005 M-Tris-HCl, pH 7.5, buffer at 4 °C, and centrifuged at 10 000 *g* for 30 min at 4 °C to remove any precipitate. Apply to a DE-52 cellulose column (20 ml in a 30 ml plastic syringe for 20–100 mg of protein) pre-washed with one litre of buffer. The sample is washed through with buffer until the absorbance at 280 nm of the eluate is < 0.1. The column is eluted with a 400 ml gradient from 0 to 0.2 M-NaCl in the same buffer. A flow rate of $\simeq 20$ ml/h is used throughout and 4 ml fractions are collected. On completion of the gradient the column is eluted with 1 M-NaCl in the same buffer until the absorbance at 280 nm of the eluate is < 0.1. The A_{280} of the fractions is measured to locate peaks of protein. Representative fractions are analysed by SDS-PAGE. Fab usually flows through or elutes in the early part of the gradient. If Fab is bound then it will often be sufficiently pure at this stage. Fc binds to the column and elutes well into the gradient. Residual IgG elutes between Fab and Fc. When Fab flows through the column, a further step of purification may be required. We have used Sephadex G-100 chromatography.

Harris *et al.* [37] used the method of Porter [1] to purify Fc from papain digests of polyclonal IgG from CBA mice. In general, this is not recommended as mouse Fc is more negative than mouse Fab whereas the reverse is true for rabbit Fc and Fab [36].

Sephadex G-100 chromatography

A 2.5×100 cm column of Sephadex G-100 equilibrated in PBS containing 0.02% NaN_3 at 4 °C is used. Samples containing 20–100 mg are concentrated if necessary and applied in a volume of 5–20 ml. The column is run at a flow rate of $\simeq 30$ ml/h and 10 ml fractions collected. Analysis is as for the DEAE column. For smaller preparations (1–20 mg), a 1.0×55 cm column of Sephadex G-100 superfine is used with samples applied in 1–2 ml. The flow rate is 2 ml/h and 1 ml fractions collected.

Protein A-Sepharose chromatography

Conditions of digestion that give optimal yields of Fab and Fc from IgG2 will often leave significant residual IgG. The simplest strategy is to deplete IgG2 and Fc with an affinity column of staphylococcal protein A [38,39].

Protein A-Sepharose from Pharmacia Fine Chemicals is described as having a capacity for IgG of 20 mg/ml. For depletion purposes we use 1 ml per 5 mg of IgG2 or 2 mg of Fc to be removed. Quantities of IgG2 and Fc are estimated from SDS-PAGE analysis.

The column is poured in a suitably sized plastic syringe and washed with > 20 column volumes of 0.02% NaN_3 0.1 M-sodium phosphate pH 8.0 (buffer).

The papain digest is either adjusted to pH 8.0 if the digestion buffer is > 7.0 or dialysed against 0.1 M-sodium phosphate, pH 8.0, if a digestion buffer of lower pH is used.

Centrifuge at 10 000 *g* for 30 min and take supernatant. Apply to protein A-Sepharose column at a flow rate of $\sim$ 0.5 ml/min. Collect fractions of 1–10 ml depending on sample size, measure A_{280} and analyse by SDS-PAGE.

Bound IgG2 and Fc can be eluted with 0.1 M-citrate pH 3.5 and collected in tubes containing sufficient 1 M-Tris-HCl, pH 8.5, to neutralize. The column should then be re-equilibrated in buffer.

If the Fab still contains IgG and Fc a second depletion may be required. Fab containing flow-through fractions can then be further purified on Sephadex G-100 if required. Fab can be separated from IgG on the same column.

We encountered some difficulty with this approach in the case of an IgG2b monoclonal antibody that resulted from a fusion with an IgG1-secreting myeloma parent. The IgG preparation contained IgG1 and hybrid molecules with one IgG1 and one IgG2b chain. After proteolytic digestion, the residual IgG and Fc of these forms did not all bind to protein A-Sepharose [27]. This is not a general problem as most fusions are now done with non-secreting myeloma parents.

Other purification procedures

Lee *et al.* [40] prepared Fab fragments from a NZB/NZW strain monoclonal antibody that binds single-stranded DNA. Their methods were based on those described by Mage [36]. A purified IgG fraction was treated with 1% (w/w) papain for 5 h at 37 °C in a

buffer of 0.25 M-NaCl, 0.001 M-EDTA, 0.025 M-2-mercaptoethanol and 0.025 M-Tris-HCl, pH 7.5. The digest was precipitated with 80% ammonium sulphate, and then dialysed against 0.01 M-Tris-HCl, pH 8.0, 0.0001 M-EDTA and applied to a 100 ml DEAE-Sephacel column. The column was eluted with a 200 ml gradient from 0 to 1 M-NaCl. Fab was in the flow-through, whereas Fc eluted towards the end of the gradient. Fab was again precipitated with 80% ammonium sulphate dialysed into 10 mM-potassium phosphate, pH 6.5, and applied to a 60 ml phosphocellulose column. This was eluted with a gradient of 0–2 M-NaCl and Fab eluted at about 0.5 M-NaCl.

Spring & Nisonoff [41] prepared Fab fragments from a variety of myeloma proteins and sera from several strains of mouse [40]. IgG was treated with 1% (w/w) papain for 18 h at 37 °C in 0.1 M-phosphate buffer, pH 7.0, containing 0.01 M-cysteine and 0.002 M-EDTA. Digests were dialysed against 0.005 M-phosphate buffer, pH 8.0, and applied to a DEAE-cellulose column equilibrated in the same buffer. Fab passed through the column which was then washed with 0.01 M-phosphate buffer, pH 8.0. Fc was then eluted with 0.5 M-phosphate buffer, pH 8.0.

pFc′ fragment

pFc′ has been isolated from IgG1 and IgG2b as a product of pepsin digestion [42]. It is a subfragment of Fc and consists of the CH3 domains of the two heavy chains. Monoclonal IgG1, IgG2a, and IgG2b were digested for 10 h, 14 h, and 12 h with pepsin, at a weight ratio of 20:1. The buffer was 0.1 M-acetate, pH 4.8, 0.1 M-cysteine. The reaction was terminated by adjusting the pH to 7.0 with 0.1 M-NaOH. Purification was on a column of Sephadex G-150 (2.6 × 85 cm) in 0.1 M-acetate, pH 4.8, followed by a DEAE cellulose column equilibrated in 0.005 M-potassium phosphate, pH 8.1, and eluted with linear gradient from 0.005 M- to 0.2 M-potassium phosphate, pH 8.1. Under the conditions used, pFc′ was obtained from IgG1 and IgG2b but not IgG2a. The presence of a relatively large concentration of cysteine (0.1 M) was required to obtain pFc′.

IgM

The fragmentation of monoclonal murine IgM has been analysed [43,44] and the patterns compared to those obtained with IgM from five other mammalian species (Fig. 14.12). Considerable species differences were observed [44]. With trypsin (IgM 7 mg/ml; trypsin 70 μg/ml; in 0.2 M-NaCl, 0.1 M-Tris-HCl, 0.01 M-$CaCl_2$, pH 8.3) at 55 °C the major products were $F(ab')_2$ and Fab. The $F(ab')_2$ fragment of IgM contains the Cμ1, Cμ2, and Cμ3 domains of the heavy chain with an H—H disulphide in the Cμ2. This yields Fab by further cleavage on the NH_2-terminal side of the disulphide bond. No Fc fragment or pentameric Fc fragments of the type obtained with IgM of other species were detected [44,45,46].

The pathway of pepsin digestion of murine IgM

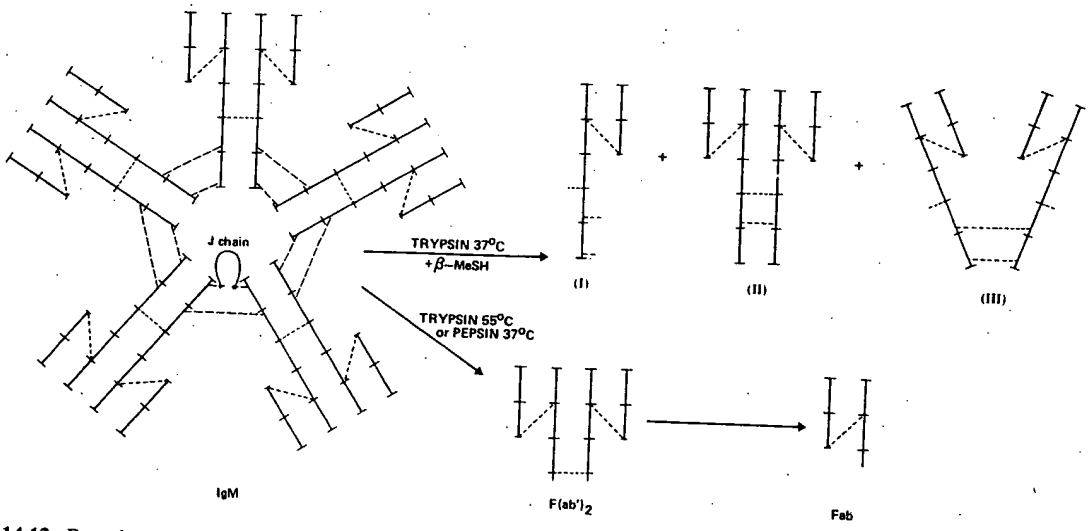


Fig. 14.12. Postulated fragmentation patterns of murine IgM [44, 47]. The arrangement of disulphides is from Kehry *et al.* [58] and is not entirely consistent with an earlier report [59]. Structures I and II could exist in forms with one of the two disulphides joining the heavy chain fragments reduced. This could account for the multiple bands on non-reducing SDS-PAGE seen by Matthew & Reichardt [47].

(IgM 7 mg/ml; pepsin 70 μg/ml; in 0.2 M-NaCl, 0.1 M-sodium acetate, pH 4.6 at 37 °C) was similar to that obtained with trypsin. After 4 h, appreciable $F(ab')_2$ was formed and this was prepared and separated from intact IgM and a small amount of Fab′ by Sephadex G-200 chromatography [44]. Fab′ was a significant product after 8 h.

Matthew & Reichardt [47] used sequential trypsin digestion and reduction of four monoclonal IgM to generate antigen binding fragments of 110 000–230 000 relative molecular mass that were, in comparison to the intact IgM, 'superior reagents in immunocytochemistry'. IgM at 1 mg/ml in 0.15 M-NaCl 0.02 M-$CaCl_2$, 0.05 M-Tris-HCl, pH 8.0, was digested with 10 μg/ml of TPCK trypsin (Millipore Co.) for 5 h at 37 °C. At this time, β-MeSH was added to a final concentration of 0.01 M and incubation continued at 37 °C. After 5 min, soybean trypsin inhibitor was added to a final concentration of 0.1 mg/ml. After 5 min further incubation at 37 °C, iodacetamide was added to a final concentration of 0.06 M and the digest left at room temperature for 10 min. Samples were then dialysed against PBS. These preparations gave a major protein peak on Biogel P-300 chromatography that corresponded to 200 000 relative molecular mass. The major peak of antigen binding activity corresponded to this protein peak. SDS-PAGE of the preparations showed polypeptides of Mr ≃ 230 000, 200 000, 180 000, and 110 000 without reduction. These all contained equal amounts of μ and κ chains. The fragment of Mr ≃ 110 000 is thought to consist of one heavy and one light chain, and the other species are thought to comprise two heavy and two light chains that are disulphide bonded in different configurations (Fig. 14.12). In a solid-phase radioimmunoassay the fragments bound to antigen with avidities that were within an order of magnitude of that obtained with intact IgM.

IgA: Fab and Fv

Fv is a univalent fragment consisting of the variable regions of the heavy and light chains. In principle, Fv is the ideal reagent for many purposes as it is small and contains the antigen combining site. In the two cases studied, the affinity of Fv and the corresponding Fab for antigen were similar (Fig. 14.13c) [48,49]. Murine Fv was first isolated from protein 315, an IgA myeloma protein possessing anti-dinitrophenol activity [49]. The first step was pepsin degradation of IgA to Fab under relatively mild conditions (IgG 8.5 mg/ml; pepsin 85 μg/ml; in 0.05 M acetate, pH 4.7, for 6 h at 37 °C). $F(ab')_2$ was not a product of this reaction.

The Fab which contained no intact IgA was separated from small peptides, pepsin, and reagents on a

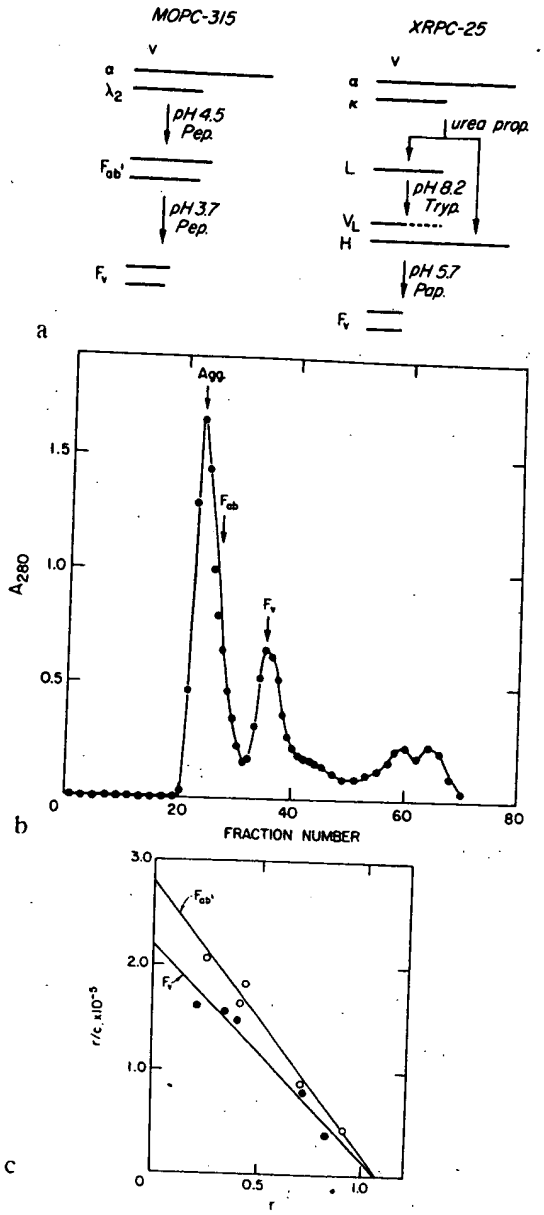


Fig. 14.13. Preparation and characterization of Fv fragments, from Sharon & Givol [49]. (a) shows the two schemes used to prepare Fv fragments. (b) shows the final stage of the preparation of Fv from XRPC-25. The papain digest of the recombined heavy chain and fragmented light chain was fractionated on a Sephadex G-75 (2.5 × 80 cm). Fractions were of 8 ml. The aggregated material was mostly small peptides. (c) shows a Scatchard plot of the results of an equilibrium dialysis experiment to measure the affinities of Fab′ and Fv of XRPC-25 for [^{3}H] DNP-lysine; r represents the number of ligand molecules that can be bound per antibody molecule; c is the free concentration of [^{3}H] DNP-lysine.

DNP-lysine-Sepharose affinity column [50]. No $F(ab')_2$ was formed, suggesting that primary pepsin cleavage is analogous to that seen with IgG2b. Fab was then digested with pepsin at pH 3.7 to yield Fv [51]. The Fab (10 mg/ml in 0.15 M-NaCl, 0.01 M-sodium phosphate, pH 7.4) was brought to pH 3.8 by the addition of one tenth the volume of 1 M-sodium acetate, pH 3.7. The solution became turbid. Pepsin at 10 mg/ml in 0.01 M-sodium acetate was added to give 100 μg/ml pepsin. Digestion was for 4 h at 37 °C and stopped by adjusting the pH to 7.0, at which point most of the precipitate dissolved. The supernatant after centrifugation was purified on DNP-lysine-Sepharose and Fv separated from residual Fab on a column of Sephadex G-75. Ninety per cent of the material eluting from the G75 column was Fv.

Hochman *et al.* [51] found that direct digestion of protein 315 IgA at pH 3.8 did not give good yields of Fv. However, a good yield was obtained when a 6 h digest at pH 4.5 was adjusted to pH 3.8, a further aliquot of pepsin (1:100 w/w) added, and digestion continued for 4 h.

The procedures developed for making Fv from protein 315 appear to be of limited applicability. More recently, Sharon & Givol [49], in describing an alternative procedure, stated that:

'Various attempts in our laboratory to prepare Fv fragments by digesting the Fab′ fragment from mouse myeloma proteins other than protein 315 were unsuccessful. Similarly digestion with papain, pepsin, or trypsin of the intact Ig, reduced and alkylated Ig, or molecules recombined from separated H and L chains, did not yield Fv fragment. A reasonable explanation for this may be that protein 315 has a unique type of L chain ($\lambda 2$) which is not present in other mouse immunoglobulins.'

This re-emphasizes the problems associated with generalizing the properties of monoclonal antibodies.

The method used for the myeloma IgA protein XRPC-25 having a κ light chain started with chemical separation of the heavy and light chains after mild reduction and alkylation of the inter-heavy chain disulphide bonds. The isolated light chain was cleaved in half with trypsin, reassociated with the heavy chain and the resulting complex digested with papain (Fig. 14.13a,b[49]). It may be of more general utility.

Separation of chains

Dissolve lyophilized IgA in 0.2 M-Tris-Cl, pH 8.2. Reduce with 0.1 M-β-MeSH, for 1 h at RT, and alkylate with 0.15 M-iodoacetamide, for 1 h at RT. Dialyse against 0.1 M-NaCl (3 changes) and add propionic acid to a concentration of 0.1 M.

Chromatograph at RT on column of Sephadex G-100 equilibrated in 4 M-urea, 1.0 M-propionic. Dialyse heavy and light chain pools exhaust against water and lyophilize. Redissolve at 10 m in water by raising the pH to 11.0 with 1 M-Na allowing protein to dissolve (1 min); follow this rapid neutralization.

Splitting of κ light chains

Light chain (10 mg/ml) was digested with TPC trypsin (w/w 1:30) in 0.1 M-Tris-HCl, pH 8.2, for min at 37°C. The reaction was terminated by addi soybean trypsin inhibitor (1:1 weight ratio wi respect to trypsin).

Recombination of H-chain with L-chain digest

Thirty-six milligrams of tryptic digest of light chain were diluted to 1 mg/ml and mixed with an equimolar amount (54 mg) of heavy chain at 1 mg/ml at pH 7.8. The mixture was incubated overnight at RT.

Papain digest of recombined heavy chain and light chain fragments

The recombined chains were dialysed against 0.01 M-sodium acetate, pH 5.7. The solution was made 0.01 M in mercaptoethanol, and papain (1:200 w/w) was added and incubated at 37 °C, for 80 min. Digestion was terminated by adding 0.015 M-iodoacetamide and raising pH to 8.2. Digest was chromatographed on a Sephadex G-75 column in PBS (Fig. 14.13). Peak of Fv was dialysed against water and lyophilized. A yield of 42% over light chains was obtained.

IgD

IgD is characteristically sensitive to trypsin cleavage in the hinge region. Both membrane IgD and detergent-solubilized IgD yield Fab and Fc. An $F(ab')_2$-like fragment is not an intermediate as cleavage is on the NH_2-terminal side of the inter-heavy chain disulphide bridge [52]. A secondary trypsin cleavage occurs between the two heavy chain domains of the Fc. Complete cleavage of membrane IgD was obtained with 30 μg/ml of trypsin (Sigma) for 1 h at 37 °C. Mouse spleen cells were at 2.5×10^7 cells/ml in glucose acetate buffer, pH 7.5 (glucose 1.0 g; $NaH_2PO_4 2H_2O$ 50 mg; K_2HPO_4 100 mg; KCl 200 mg; NaCl 7.6 g; sodium acetate 1.5 g—all per litre) [53]. A comparison of the susceptibility to papain cleavage of membrane IgD and IgM showed that IgD was significantly more susceptible and that soluble Fab fragments were formed. On solubilization with the detergent NP40, membrane IgM and IgD were both sensitive to papain

cleavage. In these studies of Vitetta & Uhr [54], radioiodinated cells (10^7/ml) were digested with 50–500 μg/ml papain (Sigma, St. Louis, MO), 10 mM-cysteine in Eagles minimal essential medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum, for 20–60 min at 37 °C in a 5% CO_2 incubator.

IgE

Perez-Montfort & Metzger compared the sensitivity to tryptic digestion of murine IgE alone and when bound to the IgE receptor from rat basophilic leukaemia cells [55]. The reactions were carried out on radioiodinated IgE with 1 mg/ml denatured horse haemoglobin as protein carrier. Trypsin was at 2% (w/w) and the buffer 0.04 M-Tris-HCl, 0.01 M-$CaCl_2$, 0.25% Triton X-100, pH 8.1. Digestion was at room temperature and resulted in formation of an $F(ab')_2$ fragment. Cleavage is believed to occur between the Cε4 and Cε3 domains and then between the Cε3 and Cε2 domains to yield a limit $F(ab')_2$ product having a heavy chain fragment Mr = 53 000 that contains both Cε1 and Cε2 domains.

Rat monoclonal antibodies

The relative sensitivity of monoclonal IgG from LOU/Ws1) rats to papain was IgG2a > IgG2c > IgG2b > IgG1 and to pepsin was IgG2c > IgG2b > IgG2a > IgG1 [56]. The properties of rat and mouse IgG1 are similar. In the absence of a thiol, $F(ab')_2$ fragments that are resistant to further enzymatic degradation are formed. The other subclasses all gave similar sized fragments with pepsin but were then further degraded. pFc′ was generated in significant yields from IgG2a and IgG2c. Small amounts of pFc′ were seen in IgG2b digests but were absent from those of IgG1.

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